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Cerebellar development in the absence of Gbx function in zebrafish ☆

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ABSTRACT

The midbrain–hindbrain boundary (MHB) is a well-known organizing center during vertebrate brain development. The MHB forms at the expression boundary of Otx2 and Gbx2, mutually repressive homeodomain transcription factors expressed in the midbrain/forebrain and anterior hindbrain, respectively. The genetic hierarchy of gene expression at the MHB is complex, involving multiple positive and negative feedback loops that result in the establishment of non-overlapping domains of Wnt1 and Fgf8 on either side of the boundary and the consequent specification of the cerebellum. The cerebellum derives from the dorsal part of the anterior-most hindbrain segment, rhombomere 1 (r1), which undergoes a distinctive morphogenesis to give rise to the cerebellar primordium within which the various cerebellar neuron types are specified. Previous studies in the mouse have shown that *Gbx2* is essential for cerebellar development. Using zebrafish mutants we show here that in the zebrafish *gbx1* and *gbx2* are required redundantly for morphogenesis of the cerebellar primordium and subsequent cerebellar differentiation, but that this requirement is alleviated by knocking down Otx. Expression of *fgf8*, *wnt1* and the entire MHB genetic program is progressively lost in *gbx1-;gbx2-* double mutants but is rescued by Otx knock-down. This rescue of the MHB genetic program depends on rescued Fgf signaling, however the rescue of cerebellar primordium morphogenesis is independent of both Gbx and Fgf. Based on our findings we propose a revised model for the role of Gbx in cerebellar development.

Introduction

The establishment of neuromeric compartments is critical for generating diversities during vertebrate brain development, and compartment boundaries can prevent cells with different fates from intermingling (Kiecker and Lumsden, 2005). The anterior–posterior axis of the vertebrate brain is divided into three neuromeric compartments, forebrain, midbrain, and hindbrain which is further divided into eight rhombomeres (r1–r8). The midbrain–hindbrain boundary (MHB) forms a deep morphological "isthmic" constriction in the neural tube, anterior to which the dorsal midbrain differentiates into the tectum; and posterior to which the dorsal r1 transforms its axis from the rostral–caudal orientation to the medial–lateral (a 90° rotation), thickens, and gives rise to the differentiated cell types of the cerebellum (Sgaier et al., 2005; Wingate, 2001; Zervas et al., 2004).

Neuromeres can be recognized early in development by their distinct transcription factor expression. Orthodenticle homolog 2 (Otx2) and Gastrulation brain homeobox 2 (Gbx2) are expressed in the anterior neural plate (forebrain and midbrain) and anterior hindbrain, respectively (Simeone et al., 1992, 1993; Wassarman et al., 1997). In mouse mutants that lack *Gbx2*, the midbrain expands posteriorly, r1 is absent, and no cerebellum forms (Wassarman et al., 1997). Conversely, eliminating neuroepithelial Otx2 function in $Otx2^{hotx1/hotx1}$ knock-ins results in an anterior expansion of hindbrain identity and a failure to specify forebrain and midbrain (Acampora et al., 1998).

Otx and Gbx are thought to promote the development of the tectum and cerebellum by positioning a powerful "isthmic organizer" (IsO) at their mutual expression boundary (Broccoli et al., 1999; Garda et al., 2001; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Millet et al., 1999). The IsO is a source of Wnt1 and Fibroblast growth factor-8 (Fgf8), which are expressed anterior and posterior to the boundary, respectively. Both Wnt1 and Fgf8 are necessary for the development of posterior midbrain and cerebellum (Chi et al., 2003; Jaszai et al., 2003; Mastick et al., 1996; McMahon and Bradley, 1990; Meyers et al., 1998; Reifers et al., 1998; Thomas and Capecchi, 1990). Establishment and maintenance of these spatially restricted cues at the IsO involves a complex set of regulatory interactions between the transcription factors Engrailed (En) and Pax2 and Fgf8 and Wnt1 themselves, a process we refer to herein as the "MHB program" (Wurst and Bally-Cuif, 2001). The MHB program is extinguished in mouse $Gbx2^{-/-}$ mutants but is recovered in $Gbx2^{-/-}$;$Otx2^{hotx1/hotx1}$

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double mutants, consistent with a role for Gbx and Otx in positioning but not specifying the IsO. However in *Gbx2*$^{-/-}$; *Otx2*$^{hotx1/hotx1}$ double mutants the spatial relationships of IsO signals are disorganized and consequently cerebellar differentiation fails to occur (Li and Joyner, 2001; Martinez-Barbera et al., 2001). These and other findings have suggested that in addition to its role in repressing Otx2 expression in r1, *Gbx2* is required directly for cerebellar morphogenesis and differentiation.

Here we address the relationship between the MHB program and cerebellar development in zebrafish. Using null mutations in Gbx1 and Gbx2 ("*gbx*-" fish) we show that r1 morphogenesis and cerebellar differentiation can occur independently of Gbx function, provided Otx function is depleted. Thus the primary function of Gbx in cerebellar development is to relieve Otx repression. In contrast to mouse *Gbx2*$^{-/-}$;*Otx2*$^{hotx1/hotx1}$ embryos, we demonstrate normal IsO organization in *gbx*-;*otxMO* zebrafish, with Wnt1 expressed anterior to Fgf8, suggesting that the rescue of cerebellum depends on Fgf8. Indeed, blocking Fgf signaling in *gbx*-;*otxMO* embryos prevents cerebellar specification and differentiation, however the morphogenetic events in r1 that give rise to the cerebellar primordium occur independently of both Gbx and Fgf8 when Otx is depleted. We present a new model for cerebellar development that requires Gbx-dependent relief of Otx inhibition of both the Fgf8-dependent MHB program and Fgf8-independent r1 morphogenesis.

Material and methods

Fish strains and genotyping

The wildtype (WT) zebrafish (*Danio rerio*) for morpholino injection experiments is *AB. Other fish lines used here are the transgenic lines *Tg*(*ptf1a:EGFP*)^{jh1} (Godinho et al., 2005), *Tg*(*olig2:DsRed2*)^{vu19} (Kucenas et al., 2008), and *Tg*(*hsp70l:dnfgfr1-EGFP*)^{pd1} (Lee et al., 2005). All fish lines were maintained under standard conditions and staged as previously described (Kimmel et al., 1995).

gbx1^{fh271} and *gbx2*^{fh253} fish were generated by TILLING (Draper et al., 2004). In order to facilitate our analysis of *gbx1*^{fh271};*gbx2*^{fh253} double mutants (referred to as "*gbx*-" for simplicity), we performed germline replacement as described (Ciruna et al., 2002) to generate viable fish with homozygous *gbx1*$^{fh271/fh271}$;*gbx2*$^{fh253/fh253}$ germlines. Crossing these germline-replaced fish to double heterozygotes (*gbx1*$^{fh271/+}$;*gbx2*$^{fh253/+}$) generates homozygous double mutants and heterozygous controls in equal numbers.

Mutant alleles were identified by PCR genotyping as follows: *gbx1*^{fh271}: forward primer 5′-CGAGAAGGAGTTTCACTGTAAGAAG and reverse primer 5′-GGTTCGATCTGTTGATGTTGACT followed by digestion with MwoI (New England Biolabs) generates a 199-bp+50-bp WT allele and a 249-bp mutant allele; *gbx2*$^{fh253/+}$: forward primer 5′-GAGCTTCTCCATGGACAGTGATTTAGATTA and reverse primer 5′-CTGTGAGGGACAGATATTTCTTACAGTGAA followed by digestion with MseI generates a 241-bp WT allele and a 210-bp+31-bp mutant allele.

Morpholino (MO) injections and Fgf signaling inhibition

1.2 ng of *otx1a* MO and *otx2* MO (Foucher et al., 2006), or 5 ng of *fgf8* MO (E2I2) (Draper et al., 2001) was injected into 1-cell stage embryos. To block Fgf signaling, tailbud stage embryos (10 h postfertilization; hpf) containing the heat-inducible *Tg*(*hsp70l:dnfgfr1-EGFP*) were incubated at 38 °C for 15 min (Lee et al., 2005). To block Fgf signaling pharmacologically, SU5402 (20 μM, Calbiochen) (Mohammadi et al., 1997) was added to 50% epiboly stage embryos (5.3 hpf) and embryos were incubated at 28 °C until fixation.

RNA in situ hybridization and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde with 1× phosphate-buffered saline (PBS) and 4% sucrose at 4 °C overnight. RNA in situ hybridization was performed as described (Thisse et al., 1993), except NBT/BCIP (Roche) and INT/BCIP (Roche) stocks were used as the Alkaline Phosphatase substrates. For immunohistochemistry, embryonic brains were dissected after fixation and antibody staining was performed as described (Waskiewicz et al., 2001). Antibodies used here were rabbit anti-Vglut1/slc17a7 (1:1000) (Bae et al., 2009) and mouse anti-Zebrin II/Aldoca (gift of Dr. Richard Hawkes, 1:150). Secondary antibodies used here were goat anti-rabbit (Invitrogen Alexa405 or Alexa488) and goat anti-mouse (Invitrogen Alexa594). Transmitted light images were taken on a Zeiss Axioplan2 and fluorescent images on a Zeiss Pascal or a Zeiss LSM700 confocal microscope.

Live imaging

Embryos were stained in CellTrace™ Bodipy (1:150, Invitrogen) in fish water containing 0.003% N-phenylthiourea (Sigma) at 28 °C overnight. Embryos with or without Bodipy staining were anesthetized by 0.4% ethyl 3-aminobenzoate methanesalforate salt (Fluka) and mounted in 2% low-melting point agarose (Gibco). Embryos were imaged on a Zeiss LSM700 confocal microscope.

Results

Zebrafish Gbx1 and Gbx2 function redundantly in cerebellar development

In the mouse *Gbx2* alone is required for cerebellum development (Millet et al., 1999; Wassarman et al., 1997). Although the mouse genome includes a *Gbx1* gene, it is not expressed in the early neural plate and is not involved in MHB development (Buckley et al., 2013; Rhinn et al., 2004). By contrast, zebrafish *gbx1* and *gbx2* are both expressed in the midbrain–anterior hindbrain territory during early development, suggesting that both Gbx genes may be involved in cerebellum development (Rhinn et al., 2003). In order to understand the roles of *gbx1* and *gbx2* in zebrafish cerebellum development, we identified null mutations in both genes by TILLING (Draper et al., 2004). We obtained nonsense mutants that truncate the proteins within the homeodomain of Gbx1 (*gbx1*^{fh271}: Q246X) and before the homeodomain of Gbx2 (*gbx2*^{fh253}: Y199X) (Fig. 1A). Zebrafish *gbx1* and *gbx2* are both essential genes since homozygous mutants fail to form a swim bladder and die during early larval stages (data not shown).

To investigate MHB development in *gbx1*^{fh271} and *gbx2*^{fh253} mutants, we examined the expression domains of *otx2* (midbrain), *eng2b* (r1 and posterior midbrain), and *egr2b* (hindbrain rhombomere 3 and 5) by RNA in situ hybridization at 22 h postfertilization (hpf) when mid-hindbrain patterning is complete and cerebellar morphogenesis has begun. We found normal *otx2*, *eng2b*, and *egr2b* expression in both *gbx1*^{fh271} and *gbx2*^{fh253} homozygous mutants compared to wildtype (WT) (Fig. 1B–D). In *gbx1*^{fh271}; *gbx2*^{fh253} double homozygous mutants, however, the r1 domain of *eng2b* is absent and the *otx2*-expressing midbrain territory expands posteriorly to r2, suggesting that Gbx1 and Gbx2 function redundantly to specify the r1 territory (Fig. 1E). Consistent with this, the expression of r1 markers *fgf8a*, *il17rd/sef1*, and *gbx2* itself

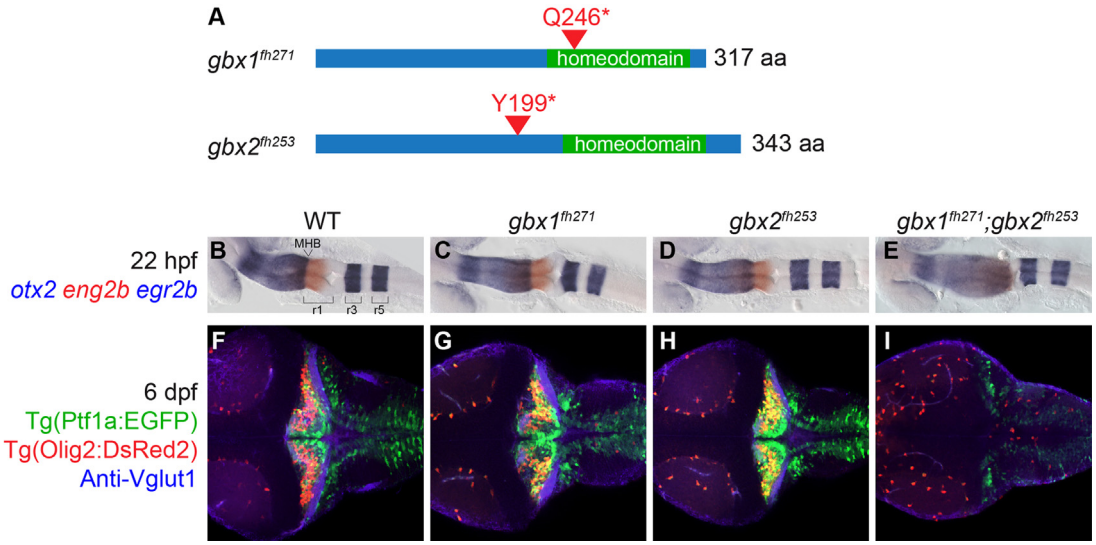

Fig. 1. Gbx1 and Gbx2 function redundantly in cerebellum development. (A) Schematic of nonsense mutations identified in zebrafish *gbx1* and *gbx2* by TILLING. Both mutations are expected to prevent DNA binding by truncating the homeodomain. (B–I) Dorsal views at 22 hpf (B–E) or 6 dpf (F–I), anterior to the left. Genotypes are shown at the top. *otx2* (blue), *eng2b* (red), and *egr2b* (blue) are expressed in midbrain, midbrain–hindbrain boundary (MHB), and rhombmere 3/5, respectively, in wildtype (WT), single and double mutants as shown. Tg(ptf1a:EGFP) (green) marks Purkinje neuron progenitors, Tg(olig2:DsRed2) (red) marks projection neurons, and anti-Vglut1/Slc17a7 (blue) marks cerebellar granule neuron axons. In $gbx1^{fh271}$;$gbx2^{fh253}$ double mutants the midbrain is expanded at the expense of r1 and no cerebellum forms.

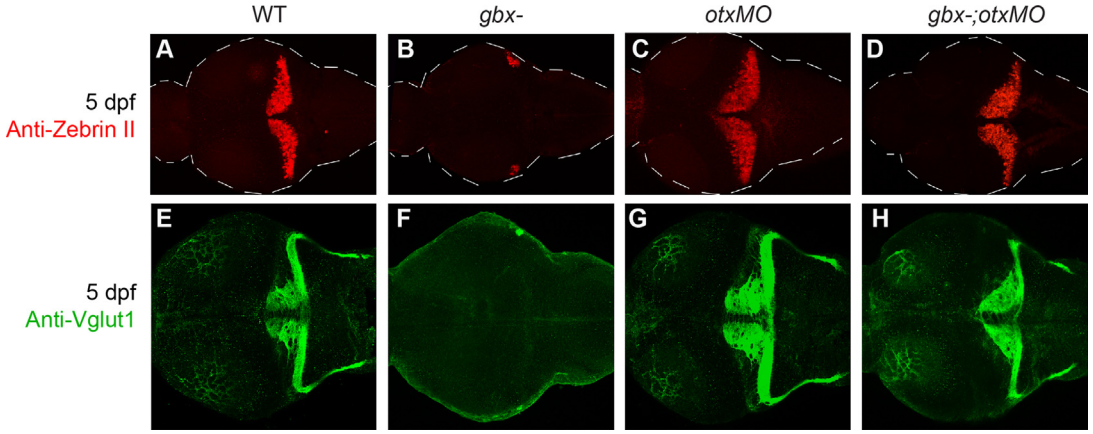

Fig. 2. Cerebellar development is rescued in *gbx*-embryos with *otx* knock-down. Dorsal views at 5 dpf, anterior is to the left. Genotypes are indicated at the top. (A–H) Zebrin II/Aldoca (A–D) and Vglut1/Slc17a7 (E–H) are expressed in cerebellar Purkinje cells and granule cell axons respectively; both are absent in *gbx*- and rescued in *otxMO* and *gbx*-;*otxMO*.

is strongly reduced or absent in $gbx1^{fh271}$;$gbx2^{fh253}$ mutants (Fig. 4B, F, and J).

We investigated cerebellar differentiation in the mutants by examining the main neuronal types of the cerebellum. In each of the single mutants the cerebellar granule cells (anti-Vglut1/slc17a7), Purkinje cells (*Tg(ptf1a:EGFP)*), and Olig2-expressing projection neurons (*Tg(olig2:DsRed2)*) were unchanged as compared to wildtype (Fig. 1F–H) (Bae et al., 2009; Elsen et al., 2009; McFarland et al., 2008; Volkmann et al., 2008). By contrast, no cerebellar differentiation occurs in $gbx1^{fh271}$;$gbx2^{fh253}$ double mutants (Fig. 1I).

We explored how the observed redundancy between *gbx1* and *gbx2* arises by measuring the distance from the posterior limit of *otx2* expression to presumptive hindbrain r3 (based on *egr2b* expression) in single and double mutants during early somite stages. The expression of zebrafish *gbx1* precedes that of *gbx2*, with *gbx1* expression being restricted to the anterior hindbrain during gastrulation (7–8 hpf) and *gbx2* expression being established only by the end of gastrulation (10 hpf) (Rhinn et al., 2003). Consistent with this, we observed a transient shortening of the anterior hindbrain in 10–12.5 hpf $gbx1^{fh271}$ single mutants that is rapidly rescued in the presence of one or two WT copies of *gbx2*, whereas this deficiency persists in $gbx1^{fh271}$;$gbx2^{fh253}$ double mutants (Fig. S1). A transient defect in anterior hindbrain development in *gbx1* morphants was also noted by Rhinn et al. (2009).

Otx is epistatic to Gbx in cerebellar development (Gbx ⊣ Otx)

The phenotype of $gbx1^{fh271}$;$gbx2^{fh253}$ double mutants resembles that of mouse $Gbx2^{-/-}$ single mutants, although the abnormalities in mouse *Gbx2* extend further into the hindbrain: mouse *Gbx2* mutants lack r2 and r3 while r3 is normal in zebrafish $gbx1^{fh271}$;$gbx2^{fh253}$ mutants and r2 is only reduced (Fig. 1E) (Millet et al., 1999; Wassarman et al., 1997). The absence of a cerebellum in $gbx1^{fh271}$;$gbx2^{fh253}$ mutants prompted us to investigate the epistatic relationship in zebrafish between Gbx and Otx, the transcription factor that normally represses cerebellum development.

We generated a hypomorphic Otx condition by knocking down both *otx1a* and *otx2* in Zebrafish (Foucher et al., 2006). In *otx1a; otx2* morphants at 22 hpf there is an expanded territory that

expresses markers of r1 identity (Figs. 3T and 4C, G, K). Consequently, an extended cerebellum develops in *otx1a*;*otx2* morphant larvae (Fig. 2C and G).

For simplicity, henceforth in this paper we refer to the *gbx1*^{fh271};*gbx2*^{fh253} double mutants simply as "*gbx* mutants" or "*gbx*-", and to the *otx1a*;*otx2* morphants simply as "*otxMO* embryos". To investigate the epistatic relationship of Gbx and Otx in cerebellar development we made *gbx*-;*otxMO* embryos. Previous work in the mouse has shown that while aspects of MHB gene expression are rescued in *Gbx2*$^{-/-}$;*Otx2*$^{hotx1/hotx1}$ double mutants relative to single mutants, the normal spatial relationships of these genes is not rescued and no cerebellar differentiation ensues (Li and Joyner, 2001; Martinez-Barbera et al., 2001). We were thus surprised to discover that in *gbx*-;*otxMO* zebrafish larva a fully formed larval cerebellum develops by 5 dpf, including Purkinje cells (Zebrin II-positive; Fig. 2D) (Lannoo et al., 1991) and granule cells (Vglut1-positive; Fig. 2H) (Bae et al., 2009). Although cerebellar Purkinje cells and granule cells were rescued in *gbx*-;*otxMO* larvae, the cerebellum did not recover to the same degree as in WT (data not shown). However, the fact that there is cerebellar differentiation demonstrates that Gbx is not strictly required for cerebellar development, and that Otx functions epistatically to Gbx. That is to say, the primary role of Gbx in zebrafish is to repress Otx-dependent repression of cerebellar development. *gbx*-;*otxMO* fish are not viable beyond 7–10 dpf, so we were unable to determine whether adult cerebellar morphogenesis was normal.

Rescue of MHB morphogenesis in gbx-;otxMO embryos

In order to understand how cerebellar development is rescued in *gbx*-;*otxMO* embryos, we investigated MHB morphogenesis during the first day of development by live imaging. In WT embryos, the MHB constriction first appears at 17 hpf due to the apico-basal shortening of cells at the boundary (Gutzman et al., 2008), and this constriction becomes more dramatic as the midbrain (III) and hindbrain (IV) ventricles rapidly inflate (arrowheads in Fig. 3A–E). These combined forces drive the opening and bilateral rotation of the r1 neuroepithelium so that by 25 hpf the cerebellar primordium (CbP) lies at 90° to the more posterior hindbrain epithelium (red lines in Fig. 3E) (Gutzman et al., 2008; Sgaier et al., 2005). The "isthmic region" where the left and right sides of the neuroepithelium contact one another derives from anterior r1. In mammals this region gives rise to the cerebellar vermis, the most medial part of the cerebellum (Sgaier et al., 2005). The length of this isthmic region can be measured in live embryos at 22 hpf, and is about 47 μm long in WT embryos (white line in Fig. 3F; $n=18$; standard deviation s.d.=4.92). During its morphogenesis the CbP thickens (white bracket in Fig. 3E). Atoh1a, a marker of cerebellar granule cell precursors, is expressed throughout the dorsal CbP and isthmic region, corresponding to the upper rhombic lip (URL) (black arrows in Fig. 3G) (Bae et al., 2009; Chaplin et al., 2010; Kani et al., 2010; Koster and Fraser, 2001).

In *gbx* mutants, a small isthmic constriction forms but is displaced posteriorly so that the isthmic region is shorter than in WT (Fig. 3M; 23 μm; $n=32$; s.d.=5.10). Hindbrain ventricle inflation causes the r1 neuroepithelium to rotate but the CbP does not thicken and no *atoh1a*-expressing URL forms (Fig. 3H–L, N). In *otxMO* embryos a constriction forms anteriorly relative to the hindbrain ventricle, resulting in an extended isthmic region and URL (Fig. 3O–U; 63 μm; $n=13$; s.d.=7.11). In *gbx*-;*otxMO* embryos a shallow constriction forms and the isthmic region is even longer (Fig. 3V–AA; 108 μm; $n=24$; s.d.=24.68). Importantly, the thickened CbP epithelium is rescued and expresses *atoh1a*, consistent with subsequent rescue of cerebellar granule cell differentiation (Figs. 3BB and 2H). Thus the morphogenesis of *gbx*-;*otxMO* embryos more closely resembles that of *otxMO* embryos than of *gbx* mutants, consistent with the epistatic relationship Gbx ⊣ Otx ⊣ cerebellum.

MHB patterning in gbx-;otxMO embryos

What is the identity of the extended isthmic region of *gbx*-;*otxMO* embryos? In WT embryos at 22 hpf *fgf8a*, *il17rd/sef1* and *gbx2* itself are all expressed specifically in the isthmic region (Fig. 4A, E, and I). Expression of all three markers is strongly reduced or absent in *gbx* mutants but is expanded in *otxMO* and even more expanded in *gbx*-;*otxMO* mutants (Fig. 4B–D, F–H, and J–L). *pax2a* and *eng1b*, which are expressed more broadly on both sides of the MHB, behave similarly (Fig. 4M–T). The rescue and expansion of the r1 domain in *gbx*-;*otxMO* embryos are detectable at earlier stages based on expression of *efnb2a* at the 8 somite stage (13 hpf; Fig. S2A–D). The rescue of all of these r1 and MHB markers in *gbx*-;*otxMO* embryos relative to *gbx*- embryos is consistent with a primary role for Gbx in repressing *otx* expression. Indeed, *otx2* is itself expressed broadly throughout the extended isthmic region of *gbx*-;*otxMO* embryos (Fig. S2H), however since translation of this mRNA is inhibited by the morpholinos, it cannot suppress the expression of the MHB program and resultant cerebellar development. We note, however, that some MHB gene expression is not rescued in *gbx*-;*otxMO* embryos, reflecting an essential requirement for Gbx in aspects of MHB development. Normally *eng1a* is expressed in a tightly restricted domain at the MHB and dorsal isthmic region (Fig. S2I). Like other MHB genes, *eng1a* expression is absent in *gbx* mutants and expanded in *otxMO* embryos, however unlike the other MHB genes described above it is not rescued in *gbx*-;*otxMO* embryos (Fig. S2J–L).

A Wnt1–Fgf8 boundary is restored in gbx-;otxMO embryos

Since cerebellar development depends on the establishment of a Wnt1–Fgf8 interface at the MHB (Broccoli et al., 1999; Millet et al., 1999), we examined *wnt1* and *fgf8* gene expression over the course of MHB development in *gbx*-;*otxMO* embryos. At the end of gastrulation (10 hpf) *wnt1* and *fgf8a* are expressed broadly on either side of the presumptive MHB, and this expression resolves into sharply defined domains over the subsequent 10 h of zebrafish development (Fig. 5A, F, and K) (Bally-Cuif et al., 1995; Reifers et al., 1998; Thisse et al., 2004).

The expression of *wnt1* and *fgf8a* in WT, *gbx*-, *otxMO* and *gbx*-;*otxMO* embryos over this time demonstrates changing requirements of the MHB program, and helps to elucidate the basis of cerebellum rescue in *gbx*-;*otxMO* embryos. At 10.33 hpf (1–3 somite stages), a broad domain of *wnt1* expression anterior to the MHB is present in *gbx*- but greatly reduced in *otxMO* and *gbx*-;*otxMO* embryos, consistent with a central role for Otx as a positive regulator of Wnt1 expression (Figs. 5B–E and S3B–D) (Foucher et al., 2006). Thus from the earliest stages, *gbx*-;*otxMO* embryos resemble *otxMO* embryos and differ from *gbx*- embryos, consistent with an epistatic role for Otx throughout MHB development (Fig. 5E, J, and O). *fgf8a* expression is essentially normal in all genotypes at this early stage, as is the initial expression of *pax2a*, consistent with independent activation of components of the MHB program (Figs. 5A–D and S3E–L) (Li and Joyner, 2001; Martinez-Barbera et al., 2001). *fgf8a* expression subsequently expands in *otxMO* and *gbx*-;*otxMO* embryos, reflecting the normal onset of repression by Otx in the midbrain (Figs. 5H, I, M, N; S3S and T). *wnt1* expression is expanded in *gbx*- embryos until the 14 hpf (9–10 somite stages) but is subsequently lost, reflecting an increasing dependence on Fgf signals from r1 (Figs. 4V; 5G, L, O; and S3N) (Chi et al., 2003). Consistent with this, in both *otxMO* and *gbx*-;*otxMO* embryos, *wnt1* expression is gradually induced within and anterior to the expanded *fgf8a* domain (Figs. 5H, M, I, N;

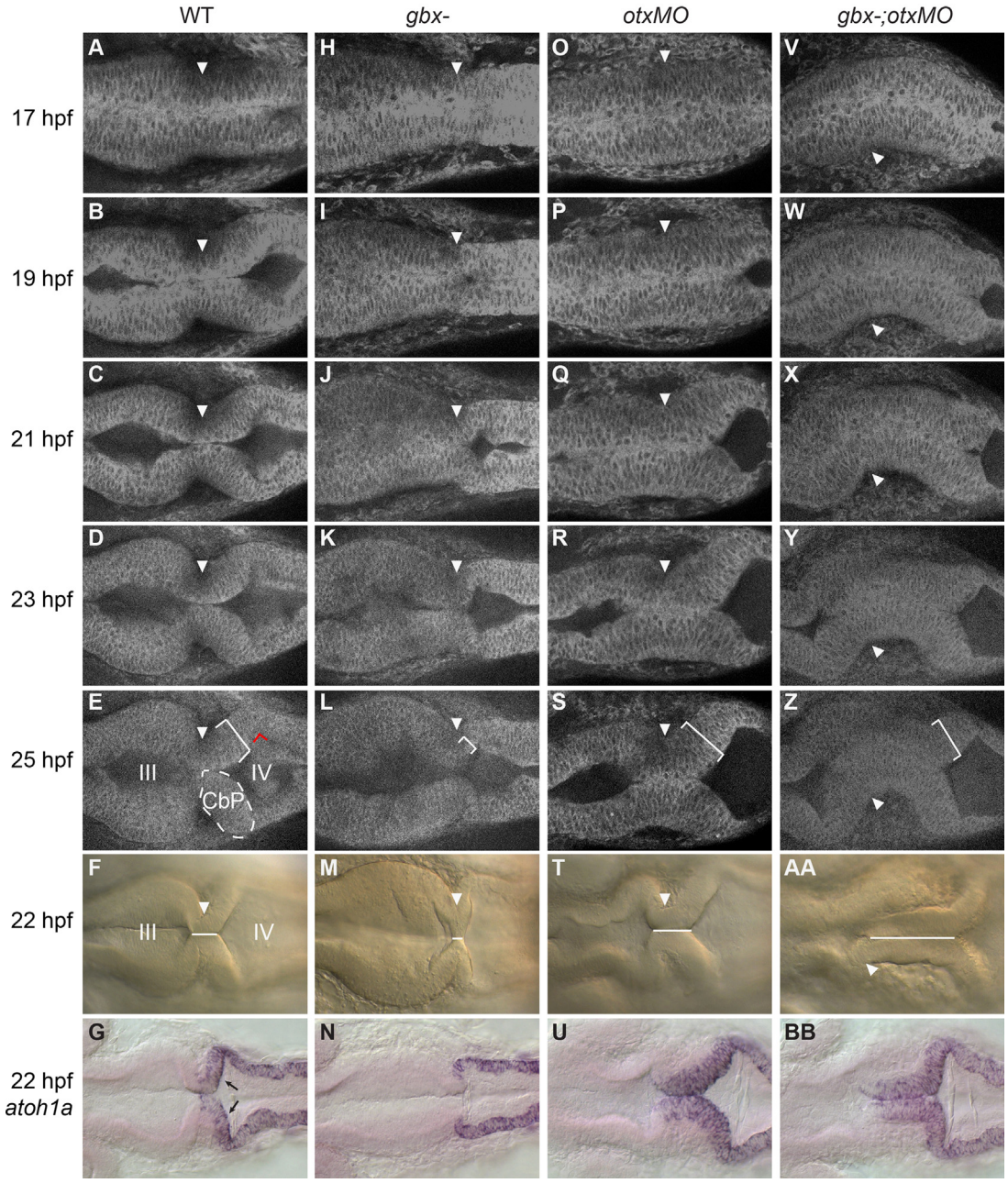

Fig. 3. Rescue of cerebellar primordium morphogenesis in *gbx*-embryos with *otx* knock-down. Timelapse of embryos during the initial stages of cerebellar morphogenesis. Dorsal views with anterior to the left; genotypes are indicated at the top. Arrowheads indicate the isthmic constriction. (A–E) In WT embryos, inflation of the midbrain (III) and hindbrain (IV) ventricles combined with cell shape changes at the MHB creates a sharp isthmic constriction flanked posteriorly by a thickened bilateral cerebellar primordium (CbP, indicated on the left side with dotted white lines and on the right side by a white bracket indicating thickness). Red lines indicate a 90° rotation of the CbP relative to the more posterior hindbrain epithelium. (H–L) Ventricle inflation is delayed in *gbx* mutants, the isthmic region is short and no thickened CbP forms. (O–S; V–Z) In *otxMO* and *gbx-;otxMO* the isthmic region is extended and a thickened CbP forms. (F, M, T, and AA) The "isthmic region" is where the left and right sides of the neuroepithelium are in contact (white line), and can be easily measured at 22 hpf in WT (F). This region is nearly absent in *gbx-* (M), but extended in *otxMO* (T) and *gbx-;otxMO* (AA). (G, N, U, and BB) *atoh1a* expression in granule cell progenitors in the upper rhombic lip (URL, black arrows) is lost in *gbx-* (N) and rescued in both *otxMO* (U) and *gbx-;otxMO* (BB).

S3O, P, S, T). As a result, the normal spatial relationship of *wnt1* and *fgf8a* is partially maintained (Figs. 4C, D, W, X; 5M and N). This is unlike mouse $Gbx2^{-/-}$;$Otx2^{hotx1/hotx1}$ double mutants, where *wnt1* and *fgf8* fully overlap throughout the anterior central nervous system (Li and Joyner, 2001; Martinez-Barbera et al., 2001). Since cerebellar development depends upon signaling between Wnt and Fgf-expressing cells (Broccoli et al., 1999; Li et al., 2002; Liu et al., 1999; Millet et al., 1999), we reason that the development of a cerebellum in *gbx-;otxMO* embryos is due to the recovery of a Wnt1–Fgf8 boundary upon Otx knock-down.

Rescue of MHB program and cerebellar differentiation but not URL specification depends on Fgf signaling in gbx-;otxMO

Rescued cerebellar development in *gbx-;otxMO* embryos correlates with rescue of the entire mid–hindbrain regulatory program including an expanded domain of Fgf signaling and the re-establishment of a Wnt1–Fgf8 boundary. We tested whether cerebellar development in *gbx-;otxMO* embryos requires Fgf by blocking Fgf signaling either using a heat-inducible dominant-negative Fgf receptor (*Tg*(*hsp70l:dnfgfr1-EGFP*)) (Lee et al., 2005),

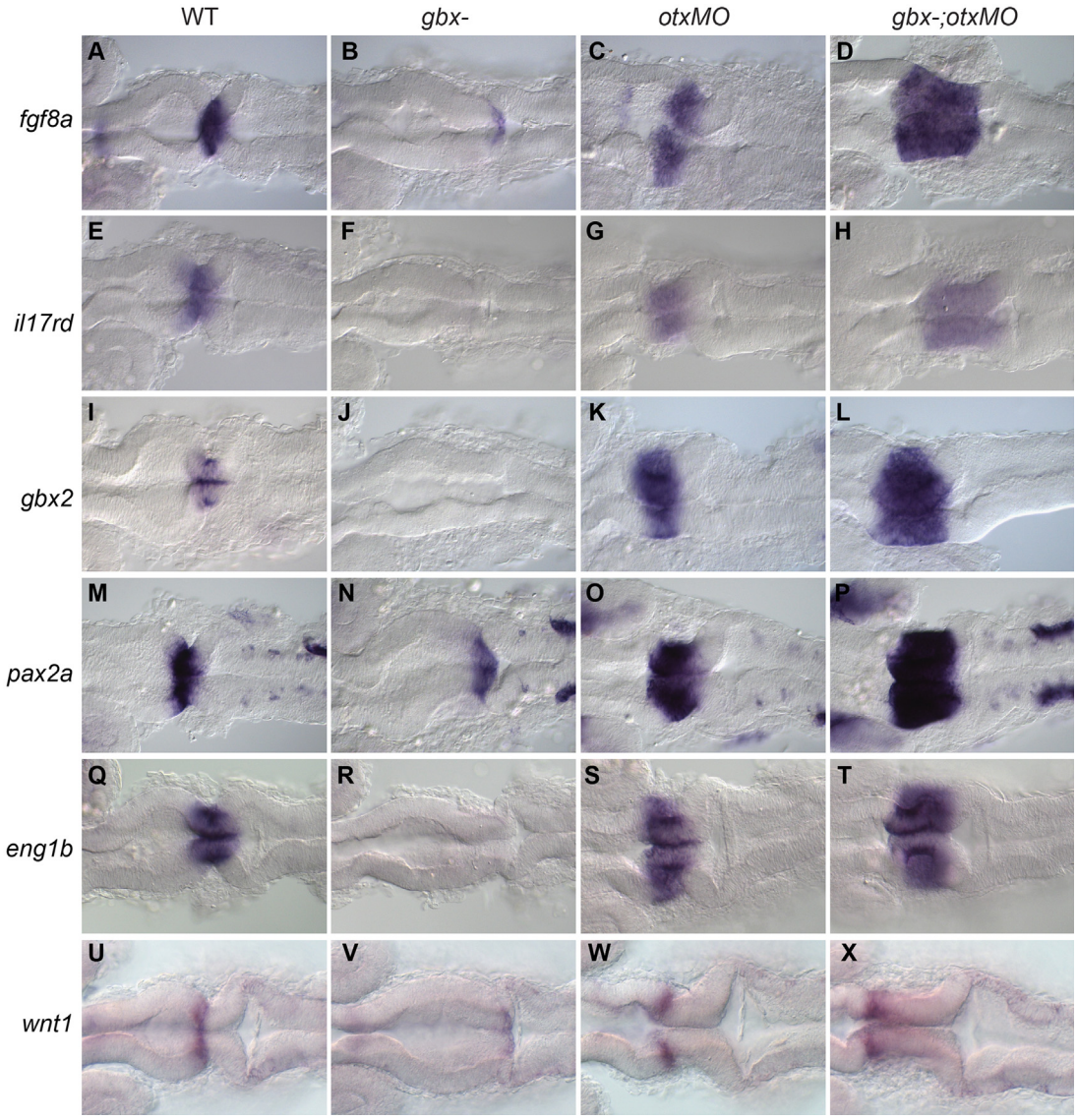

Fig. 4. Rescue of the MHB program in *gbx*-embryos with *otx* knock-down. RNA in situ hybridization with genes shown on left; genotypes indicated at the top. Dorsal views of 22 hpf embryos with anterior to the left. (A–T) *fgf8a*, *il17rd/sef*, *gbx2*, *pax2a* and *eng1b* are all expressed at or around the MHB, are absent or strongly reduced in *gbx*- but are expanded in both *otxMO* and *gbx*-;*otxMO* embryos. (U–X) *wnt1* is normally expressed in a narrow domain anterior to the MHB (U). This expression is reduced in *gbx*- (V) but rescued anterior to the extended isthmic region in *otxMO* (W) and *gbx*-;*otxMO* (X).

fgf8 morpholinos (Draper et al., 2001), or the Fgf-specific inhibitor SU5402 (Mohammadi et al., 1997). In wildtype embryos all three treatments result in a posteriorly expanded midbrain, loss of MHB markers and loss of an *atoh1a*-expressing URL, similar to *fgf8a* mutants and to *gbx* mutants (Figs. 6B and F; S4B and F; data not shown) (Jaszai et al., 2003).

As described previously (Foucher et al., 2006) knock-down of Otx in the absence of Fgf8 signaling rescues the morphogenesis of a thickened CbP and an *atoh1a*-expressing URL (Figs. 6C and S4C). This is similar to the rescue of the CbP and *atoh1a* expression that we observe upon knock-down of Otx in *gbx* mutants (Fig. 3BB). Interestingly, a thickened CbP with an *atoh1a*-expressing URL is still rescued when Otx is knocked down in embryos lacking both Gbx and Fgf signaling, demonstrating that both Gbx and Fgf function to repress Otx-dependent repression of these events in cerebellar development (Figs. 6D and S4D). However, unlike in *gbx*-;*otxMO* embryos, CbP morphogenesis and URL specification is insufficient for subsequent cerebellar development in the absence of Fgf signaling, irrespective of the presence or absence of Otx and/or Gbx. The MHB program is not rescued, the isthmic region opens and the midbrain and hindbrain ventricles fuse into a single ballooning ventricle (Figs. 6E–H and S4E–L, compare to Fig. 3Z). While the differentiation of a lateral population of zebrin+ cerebellar Purkinje cells was partially rescued in *dnFgfr*-expressing larvae upon Otx knock-down (Fig. 6K) (Foucher et al., 2006), no zebrin expression was detected in *gbx*-;*otxMO*;*dnFgfr* larvae (Fig. 6L). Thus the cerebellar rescue we observe in *gbx*-;*otxMO* larvae is entirely dependent on expanded Fgf signaling. We conclude that initial CbP morphogenesis and URL specification can occur independently of Fgf8 and Gbx provided Otx activity is knocked down, but that MHB program and subsequent robust cerebellar differentiation can all occur independently of Gbx but not of Fgf8 (Fig. 7).

Discussion

Early cerebellar development involves the induction and positioning of the IsO and the specification and morphogenesis of a cerebellar primordium (CbP) that is competent to generate

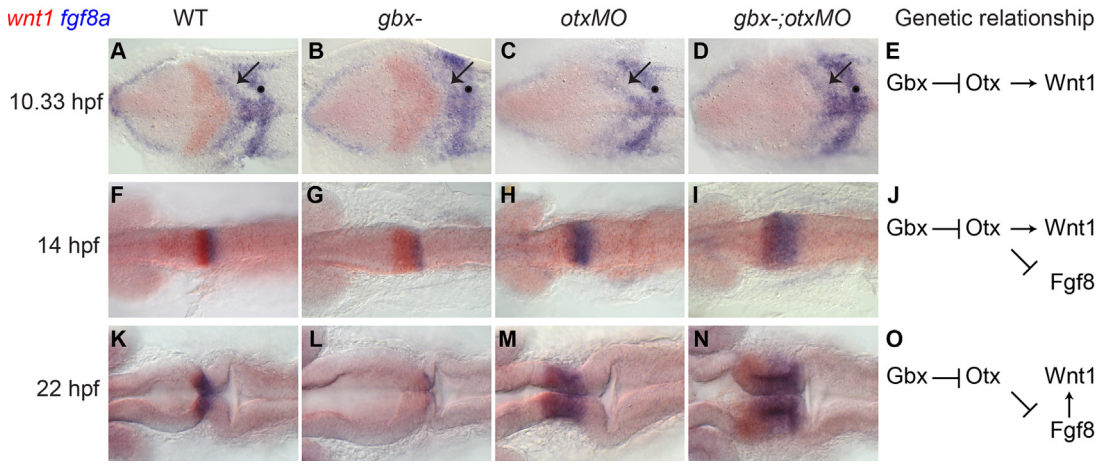

Fig. 5. Changing requirements for *wnt1* and *fgf8* expression leads to rescue of a *wnt1–fgf8* boundary in *gbx-;otxMO* embryos. RNA in situ hybridizations with *wnt1* (red) and *fgf8a* (blue) at the stages shown on the left; genotypes indicated at the top. Dorsal views with anterior to the left. (A–D, F–I, and K–N) At 10.33 hpf (1–3 somites) in WT (A), *wnt1* (red) and *fgf8a* (blue) are expressed in broad domains anterior and posterior to the presumptive MHB respectively (black arrow indicates the *fgf8a* domain in the anterior hindbrain). *fgf8a* is also expressed in hindbrain rhombomere 4 (black dot). In *gbx*-embryos *wnt1* is initially expanded (B and G) but subsequently lost (L). Conversely, in *otxMO* and *gbx-;otxMO*, *wnt1* expression is initially reduced (C and D) but subsequently recovers while the *fgf8a* domain expands (H, I, M, and N). (E, J, and O) Genetic pathways indicate the decreasing dependence of Wnt1 expression on Otx in the midbrain and its increasing dependence on Fgf8 signaling from r1.

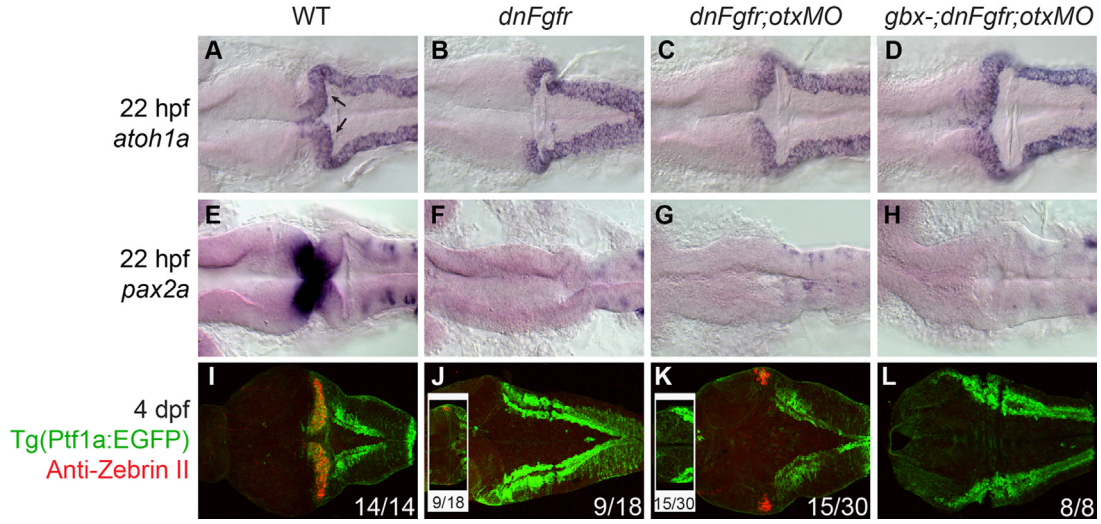

Fig. 6. Fgf signaling is required for rescue of the MHB program and cerebellar differentiation but not URL specification in *gbx-;otxMO*. Dorsal views of 22 hpf embryos (A–H) or 4 dpf larvae (I–L) with anterior to the left. Genotypes are indicated at the top. (A–H) RNA in situ hybridizations with genes indicated on left. *atoh1a* expression in the URL (black arrows in A) is absent in *dnFgfr* (B) but is rescued in *dnFgfr;otxMO* (C) and *gbx-;dnFgfr;otxMO* embryos (D). By contrast, MHB gene expression (*pax2a*, E) is not rescued by *otx* knock-down in the absence of Fgf signaling (G and H). (I–L) Zebrin II/Aldoca (red) normally expresses in cerebellar Purkinje cells and Tg(ptf1a:EGFP) (green) marks Purkinje neuron progenitors in WT (I; 14 larvae examined), but zebrin expression is absent and Tg(ptf1a:EGFP) is only expressed in lower rhombic lip in *gbx-;dnFgfr;otxMO* (L; 8 larvae examined). In *dnFgfr* (J) larvae, zebrin expression is absent (9 out of 18 larvae) or few zebrin-expressing cells are lying laterally at the junction of the tectum and the ptf1a:EGFP-expressing lower rhombic lip (the box in J; 9 out of 18). Similar to *dnFgfr* larvae, zebrin expression is either in the lateral junction between the tectum and lower rhombic lip in *dnFgfr;otxMO* (K; 15 out of 30 larvae) or is absent (the box in K).

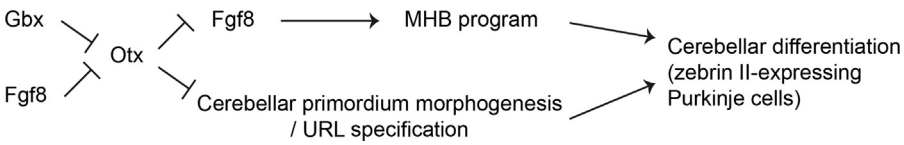

Fig. 7. A model for cerebellar morphogenesis. Our results suggest that normal cerebellar differentiation results from both the expression of the MHB program and the morphogenesis of the cerebellar primordium where cerebellar cell fates arise. Although our focus here has been on the URL, the CbP also includes the generative zone for Purkinje and projection neuron progenitors. Gbx and Fgf8 promote both processes by inhibiting their repression by Otx. Additionally, Fgf8 is required for the execution of the MHB program itself. Thus CbP morphogenesis and URL specification are both rescued in the absence of Gbx or Fgf8 signaling (or both) by Otx knock-down, while the MHB program and further cerebellar differentiation in Otx knock-down embryos requires Fgf8 but not Gbx.

cerebellar neurons. We find that in zebrafish the combined activity of Gbx1 and Gbx2 is required for both of these events, however this requirement is indirect — via the inhibition of Otx activity. When Gbx function is eliminated in a background of reduced Otx activity, IsO induction and formation of the cerebellar primordium are both rescued and apparently normal cerebellar histogenesis ensues. Rescue of cerebellar development in *gbx-;otxMO* embryos depends on rescue of Fgf8 expression, however initial CbP morphogenesis and URL specification can occur independent of either Gbx or Fgf8. We propose a new model for cerebellar development

in which Otx functions epistatically to Gbx and Fgf8 in CbP morphogenesis and URL specification while Fgf8 also functions epistatically to Otx in driving the MHB program (Fig. 7).

Gbx1 and Gbx2 function redundantly in cerebellar development in the fish

Based on gene expression patterns, Rhinn et al. (2003) predicted that zebrafish *gbx1* and *gbx2* genes may have overlapping functions in cerebellum development, with *gbx1* functioning earlier than *gbx2*. Consistent with this, careful analysis of *gbx1* morphant zebrafish identified a subtle and transient defect in the anterior hindbrain development (Rhinn et al., 2009). Two other reports (Burroughs-Garcia et al., 2011; Kikuta et al., 2003) described dramatic defects in the development of the anterior hindbrain in *gbx2* morphant zebrafish, suggestive of a strong independent requirement for *gbx2* akin to the situation in the mouse. By making genetic null mutations in both genes we have now demonstrated that *gbx2* expression compensates for the lack of *gbx1* in the Zebrafish as predicted by Rhinn et al. (2003, 2009). In contrast, the morphological abnormalities and cell death observed in *gbx2* morphants is likely attributable to morpholino toxicity.

Otx is epistatic to Gbx in cerebellar development

A role for Gbx in repressing Otx is well established from gain- and loss-of-function studies in chick and mouse (Liu and Joyner, 2001). However a number of lines of evidence have suggested that Gbx has additional functions that are not attributable to Otx repression. First among these is the finding that cerebellar development is not rescued in $Gbx2^{-/-}$;$Otx2^{hotx1/hotx1}$ double mutant mice (Li and Joyner, 2001; Martinez-Barbera et al., 2001). Although the genes that make up the MHB program are expressed in these mice, they are deployed in a disorganized manner, so that Wnt1 and Fgf8, the key inductive signals that are normally expressed on either side of the MHB, are co-expressed. This implied that in addition to repressing Otx, Gbx has a separate role in repressing Wnt1 in the anterior hindbrain and thereby maintaining the normal complementary domains of Wnt1 and Fgf8 (Li et al., 2002). We find that in *gbx*-;*otxMO* zebrafish, while the domains of *wnt1* and *fgf8* are expanded and their boundaries are diffuse, their normal spatial domains are retained, with *wnt1* being expressed anterior to *fgf8*. We reason that this rescue of a Wnt1–Fgf8 boundary in *gbx*-;*otxMO* embryos leads to rescued cerebellar morphogenesis.

How do we account for this difference between the mouse $Gbx2^{-/-}$;$Otx2^{hotx1/hotx1}$ phenotype and the zebrafish *gbx*-;*otxMO* phenotype? In both the $Gbx2^{-/-}$ mouse and the $gbx1^{-/-}$;$gbx2^{-/-}$ fish, Gbx function in the brain is expected to be entirely abrogated. In contrast, while the mouse $Otx2^{hotx1/hotx1}$ eliminates Otx function in the brain, the zebrafish *otxMO* phenotype is hypomorphic due both to the incomplete nature of MO knock-down and to the existence of a third *otx* gene, *otx1b* (Mercier et al., 1995). As a result, whereas mouse $Otx2^{hotx1/hotx1}$ mutants lack a midbrain and forebrain, zebrafish *otxMO* embryos lack a midbrain but the forebrain is intact (Acampora et al., 1998; Foucher et al., 2006; Scholpp et al., 2007). One possibility is that the low level of Otx activity that persists in *gbx*-;*otxMO* embryos is sufficient to generate an Otx/non-Otx boundary at a new diencephalon-hindbrain junction. Since Otx promotes Wnt1 expression and represses Fgf8 expression, this boundary is in turn sufficient to generate the Wnt1–Fgf8 boundary essential for cerebellar development. This does not happen in the absence of *gbx* alone because the expanded domain of *otx* expression in *gbx*- embryos engulfs the entire MHB competence region and extinguishes the Wnt1–Fgf8 feedback loop that would normally emerge there. This predicts that cerebellar development would similarly be rescued in mouse *Gbx2* mutants if *Otx2* levels were reduced but not eliminated. Cerebellar development is not rescued in strongly hypomorphic $Otx^{\lambda/-}$ mutants in which Gbx2 activity is eliminated (Martinez-Barbera et al., 2001), and more weakly hypomorphic Otx conditions, such as the $Otx1^{+/-}$;$Otx2^{+/-}$ mutants that more closely resemble the *otxMO* phenotype (Suda et al., 1997) have not been tested in the context of *Gbx2* loss-of-function.

Another difference is in the *gbx* loss-of-function phenotype itself. Whereas mouse *Gbx2* mutants lack rhombomeres 1–3, our zebrafish *gbx* mutants lack only r1 (Li and Joyner, 2001; Martinez-Barbera et al., 2001; Millet et al., 1999; Wassarman et al., 1997). This broader requirement for Gbx2 in the mouse hindbrain corresponds with posteriorly shifted and expanded domain of MHB gene expression at early stages in mouse *Gbx2* mutants whereas in zebrafish *gbx* mutants MHB gene expression is initiated but rapidly extinguished (Li and Joyner, 2001; Martinez-Barbera et al., 2001; Millet et al., 1999). This more limited requirement for Gbx in the fish could reflect a narrower "MHB competence" domain (Li and Joyner, 2001) that is more easily engulfed by expanded Otx expression in *gbx* mutants, or it could reflect more restricted signals that induce the MHB program.

Although cerebellar development is rescued to a remarkable degree in *gbx*-;*otxMO* embryos consistent with the epistatic relationship gbx ⊣ otx ⊣ cerebellum, it should be noted that *gbx*-;*otxMO* embryos are not identical to *otxMO* embryos, indicating independent functions for Gbx as well. The isthmic region that is expanded in *otxMO* embryos is even more expanded in *gbx*-;*otxMO* embryo, suggesting direct repression of the program by Gbx. Conversely, the normally tightly MHB-restricted domain of *eng1a* is absent in both *gbx*- and *gbx*-;*otxMO* embryos, suggesting an Otx-independent positive requirement for *gbx* in *eng1a* expression. Thus although cerebellar specification and morphogenesis can occur in *gbx*-;*otxMO* embryos, its patterning and neuronal organization are unlikely to be entirely normal.

The dual role of Fgf8 in cerebellar specification and morphogenesis

If a rescued *wnt1–fgf8* boundary in *gbx*-;*otxMO* embryos is responsible for the rescue of cerebellar specification and morphogenesis, cerebellar rescue should require Fgf8 signaling. Indeed, MHB gene expression and subsequent cerebellar morphogenesis and histogenesis is disrupted in *gbx*-;*otxMO* embryos when Fgf signaling is blocked, as it is in WT and *otxMO* embryos when Fgf signaling is blocked. Foucher et al. (2006) described rescue of an *atoh1a*-expressing upper rhombic lip (URL) in zebrafish *fgf8* mutant embryos in which *otx* is knocked down. The URL is induced within the cerebellar primordium by the interaction between r1 neuroepithelium and roofplate ectoderm, and the *atoh1a*-expressing cells there are cerebellar granule cell progenitors (Wingate, 2001). In both *fgf8* mutants and *gbx*-embryos no cerebellar primordium or URL forms. We confirmed URL rescue by *otx* knock-down in embryos in which Fgf signaling is blocked and also showed that this rescue is independent of, or even enhanced by, loss of *gbx* function. However no IsO formed in *otxMO* or *gbx*-;*otxMO* embryos without Fgf signaling, and subsequent cerebellar differentiation was failed. This is in contrast to *gbx*-;*otxMO* embryos in which an IsO, a cerebellar primordium and cerebellar neuron differentiation are all robustly rescued. We conclude that initial cerebellar primordium morphogenesis and specification of the URL requires neither Gbx nor Fgf8 provided Otx activity is reduced. Stated differently, Gbx and Fgf8 both function to prevent Otx-dependent inhibition of URL specification. However Fgf8, unlike Gbx, is also required as a key component of

the MHB gene expression program that is required for subsequent cerebellar differentiation (Fig. 7).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.026.

References

Acampora, D., Avantaggiato, V., Tuorto, F., Briata, P., Corte, G., Simeone, A., 1998. Visceral endoderm-restricted translation of Otx1 mediates recovery of Otx2 requirements for specification of anterior neural plate and normal gastrulation. Development 125, 5091–5104.

Bae, Y.K., Kani, S., Shimizu, T., Tanabe, K., Nojima, H., Kimura, Y., Higashijima, S., Hibi, M., 2009. Anatomy of zebrafish cerebellum and screen for mutations affecting its development. Dev. Biol. 330, 406–426.

Bally-Cuif, L., Cholley, B., Wassef, M., 1995. Involvement of Wnt-1 in the formation of the mes/metencephalic boundary. Mech. Dev. 53, 23–34.

Broccoli, V., Boncinelli, E., Wurst, W., 1999. The caudal limit of Otx2 expression positions the isthmic organizer. Nature 401, 164–168.

Buckley, D.M., Burroughs-Garcia, J., Lewandoski, M., Waters, S.T., 2013. Characterization of the Gbx1−/− mouse mutant: a requirement for Gbx1 in normal locomotion and sensorimotor circuit development. PLoS One 8, e56214.

Burroughs-Garcia, J., Sittaramane, V., Chandrasekhar, A., Waters, S.T., 2011. Evolutionarily conserved function of Gbx2 in anterior hindbrain development. Dev. Dyn. 240, 828–838.

Chaplin, N., Tendeng, C., Wingate, R.J., 2010. Absence of an external germinal layer in zebrafish and shark reveals a distinct, anamniote ground plan of cerebellum development. J. Neurosci. 30, 3048–3057.

Chi, C.L., Martinez, S., Wurst, W., Martin, G.R., 2003. The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum. Development 130, 2633–2644.

Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., Schier, A.F., 2002. Production of maternal-zygotic mutant zebrafish by germ-line replacement. Proc. Natl. Acad. Sci. USA 99, 14919–14924.

Draper, B.W., McCallum, C.M., Stout, J.L., Slade, A.J., Moens, C.B., 2004. A high-throughput method for identifying N-ethyl-N-nitrosourea (ENU)-induced point mutations in zebrafish. Methods Cell Biol. 77, 91–112.

Draper, B.W., Morcos, P.A., Kimmel, C.B., 2001. Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. Genesis 30, 154–156.

Elsen, G.E., Choi, L.Y., Prince, V.E., Ho, R.K., 2009. The autism susceptibility gene met regulates zebrafish cerebellar development and facial motor neuron migration. Dev. Biol. 335, 78–92.

Foucher, I., Mione, M., Simeone, A., Acampora, D., Bally-Cuif, L., Houart, C., 2006. Differentiation of cerebellar cell identities in absence of Fgf signalling in zebrafish Otx morphants. Development 133, 1891–1900.

Garda, A.L., Echevarria, D., Martinez, S., 2001. Neuroepithelial co-expression of Gbx2 and Otx2 precedes Fgf8 expression in the isthmic organizer. Mech. Dev. 101, 111–118.

Godinho, L., Mumm, J.S., Williams, P.R., Schroeter, E.H., Koerber, A., Park, S.W., Leach, S.D., Wong, R.O., 2005. Targeting of amacrine cell neurites to appropriate synaptic laminae in the developing zebrafish retina. Development 132, 5069–5079.

Gutzman, J.H., Graeden, E.G., Lowery, L.A., Holley, H.S., Sive, H., 2008. Formation of the zebrafish midbrain–hindbrain boundary constriction requires laminin-dependent basal constriction. Mech. Dev. 125, 974–983.

Jaszai, J., Reifers, F., Picker, A., Langenberg, T., Brand, M., 2003. Isthmus-to-midbrain transformation in the absence of midbrain–hindbrain organizer activity. Development 130, 6611–6623.

Kani, S., Bae, Y.K., Shimizu, T., Tanabe, K., Satou, C., Parsons, M.J., Scott, E., Higashijima, S., Hibi, M., 2010. Proneural gene-linked neurogenesis in zebrafish cerebellum. Dev. Biol. 343, 1–17.

Kiecker, C., Lumsden, A., 2005. Compartments and their boundaries in vertebrate brain development. Nat. Rev. Neurosci. 6, 553–564.

Kikuta, H., Kanai, M., Ito, Y., Yamasu, K., 2003. gbx2 Homeobox gene is required for the maintenance of the isthmic region in the zebrafish embryonic brain. Dev. Dyn. 228, 433–450.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Koster, R.W., Fraser, S.E., 2001. Direct imaging of in vivo neuronal migration in the developing cerebellum. Curr. Biol. 11, 1858–1863.

Kucenas, S., Takada, N., Park, H.C., Woodruff, E., Broadie, K., Appel, B., 2008. CNS-derived glia ensheath peripheral nerves and mediate motor root development. Nat. Neurosci. 11, 143–151.

Lannoo, M.J., Ross, L., Maler, L., Hawkes, R., 1991. Development of the cerebellum and its extracerebellar Purkinje cell projection in teleost fishes as determined by zebrin II immunocytochemistry. Prog. Neurobiol. 37, 329–363.

Lee, Y., Grill, S., Sanchez, A., Murphy-Ryan, M., Poss, K.D., 2005. Fgf signaling instructs position-dependent growth rate during zebrafish fin regeneration. Development 132, 5173–5183.

Li, J.Y., Joyner, A.L., 2001. Otx2 and Gbx2 are required for refinement and not induction of mid–hindbrain gene expression. Development 128, 4979–4991.

Li, J.Y., Lao, Z., Joyner, A.L., 2002. Changing requirements for Gbx2 in development of the cerebellum and maintenance of the mid/hindbrain organizer. Neuron 36, 31–43.

Liu, A., Joyner, A.L., 2001. Early anterior/posterior patterning of the midbrain and cerebellum. Annu. Rev. Neurosci. 24, 869–896.

Liu, A., Losos, K., Joyner, A.L., 1999. FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. Development 126, 4827–4838.

Martinez-Barbera, J.P., Signore, M., Boyl, P.P., Puelles, E., Acampora, D., Gogoi, R., Schubert, F., Lumsden, A., Simeone, A., 2001. Regionalisation of anterior neuroectoderm and its competence in responding to forebrain and midbrain inducing activities depend on mutual antagonism between OTX2 and GBX2. Development 128, 4789–4800.

Mastick, G.S., Fan, C.M., Tessier-Lavigne, M., Serbedzija, G.N., McMahon, A.P., Easter Jr., S.S., 1996. Early deletion of neuromeres in Wnt-1−/− mutant mice: evaluation by morphological and molecular markers. J. Comp. Neurol. 374, 246–258.

McFarland, K.A., Topczewska, J.M., Weidinger, G., Dorsky, R.I., Appel, B., 2008. Hh and Wnt signaling regulate formation of olig2+ neurons in the zebrafish cerebellum. Dev. Biol. 318, 162–171.

McMahon, A.P., Bradley, A., 1990. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 62, 1073–1085.

Mercier, P., Simeone, A., Cotelli, F., Boncinelli, E., 1995. Expression pattern of two otx genes suggests a role in specifying anterior body structures in zebrafish. Int. J. Dev. Biol. 39, 559–573.

Meyers, E.N., Lewandoski, M., Martin, G.R., 1998. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat. Genet. 18, 136–141.

Millet, S., Campbell, K., Epstein, D.J., Losos, K., Harris, E., Joyner, A.L., 1999. A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. Nature 401, 161–164.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B.K., Hubbard, S.R., Schlessinger, J., 1997. Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 276, 955–960.

Reifers, F., Bohli, H., Walsh, E.C., Crossley, P.H., Stainier, D.Y., Brand, M., 1998. Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain–hindbrain boundary development and somitogenesis. Development 125, 2381–2395.

Rhinn, M., Lun, K., Ahrendt, R., Geffarth, M., Brand, M., 2009. Zebrafish gbx1 refines the midbrain–hindbrain boundary border and mediates the Wnt8 posteriorization signal. Neural Dev. 4, 12.

Rhinn, M., Lun, K., Amores, A., Yan, Y.L., Postlethwait, J.H., Brand, M., 2003. Cloning, expression and relationship of zebrafish gbx1 and gbx2 genes to Fgf signaling. Mech. Dev. 120, 919–936.

Rhinn, M., Lun, K., Werner, M., Simeone, A., Brand, M., 2004. Isolation and expression of the homeobox gene Gbx1 during mouse development. Dev. Dyn. 229, 334–339.

Scholpp, S., Foucher, I., Staudt, N., Peukert, D., Lumsden, A., Houart, C., 2007. Otx1l, Otx2 and Irx1b establish and position the ZLI in the diencephalon. Development 134, 3167–3176.

Sgaier, S.K., Millet, S., Villanueva, M.P., Berenshteyn, F., Song, C., Joyner, A.L., 2005. Morphogenetic and cellular movements that shape the mouse cerebellum; insights from genetic fate mapping. Neuron 45, 27–40.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A., Boncinelli, E., 1992. Nested expression domains of four homeobox genes in developing rostral brain. Nature 358, 687–690.

Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M.R., Nigro, V., Boncinelli, E., 1993. A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. EMBO J. 12, 2735–2747.

Suda, Y., Matsuo, I., Aizawa, S., 1997. Cooperation between Otx1 and Otx2 genes in developmental patterning of rostral brain. Mech. Dev. 69, 125–141.

Thisse, B., Heyer, V., Lux, A., Alunni, V., Degrave, A., Seiliez, I., Kirchner, J., Parkhill, J.P., Thisse, C., 2004. Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. Methods Cell. Biol. 77, 505–519.

Thisse, C., Thisse, B., Schilling, T.F., Postlethwait, J.H., 1993. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 119, 1203–1215.
Thomas, K.R., Capecchi, M.R., 1990. Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature 346, 847–850.
Volkmann, K., Rieger, S., Babaryka, A., Koster, R.W., 2008. The zebrafish cerebellar rhombic lip is spatially patterned in producing granule cell populations of different functional compartments. Dev. Biol. 313, 167–180.
Waskiewicz, A.J., Rikhof, H.A., Hernandez, R.E., Moens, C.B., 2001. Zebrafish Meis functions to stabilize Pbx proteins and regulate hindbrain patterning. Development 128, 4139–4151.
Wassarman, K.M., Lewandoski, M., Campbell, K., Joyner, A.L., Rubenstein, J.L., Martinez, S., Martin, G.R., 1997. Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. Development 124, 2923–2934.
Wingate, R.J., 2001. The rhombic lip and early cerebellar development. Curr. Opin. Neurobiol. 11, 82–88.
Wurst, W., Bally-Cuif, L., 2001. Neural plate patterning: upstream and downstream of the isthmic organizer. Nat. Rev. Neurosci. 2, 99–108.
Zervas, M., Millet, S., Ahn, S., Joyner, A.L., 2004. Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1. Neuron 43, 345–357.